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TRADITIONAL INDIAN HERBAL EXTRACTS USED *IN VITRO* AGAINST GROWTH OF THE PATHOGENIC BACTERIA – *AEROMONAS HYDROPHILA*

R. Bhuvaneswari and C. Balasundaram*

Fish Disease Diagnostic Laboratory, Department of Animal Science, School of Life Science, Bharathidasan University, Tiruchirappalli, Tamilnadu, India

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Abstract

Crude ethanol extracts of *Acalypha indica, Acorus calamus, Coleus aromaticus, Heliotropium indicum*, and *Indigofera aspalathoides* were screened for antibacterial activity *in vitro* against the growth of the fish pathogenic bacteria, *Aeromonas hydrophila*. Terramycin, widely used to control *A. hydrophila* in aquaculture, was used as a positive control. The herbs *A. calamus* and *I. aspalathoides* warded off the growth of the pathogen completely at minimum inhibitory concentrations of 1.29 and 2.16 mg/l, respectively. A minimum bacterial concentration of 1.00 cfu occurred at concentrations of 0.77 mg/ml for *A. calamus* and 1.29 mg/ml for *I. aspalathoides*. The order of potency of the herbs in warding off growth of *A. hydrophila in vitro* was ranked: *A. calamus, I. aspalathoides, C. aromaticus, A. indica*, and *H. indicum.* The inhibitory potency of *A. calamus* and *I. aspalathoides* was significantly higher ($p<0.05$) than that of the positive control, indicating the potential of these herbs to replace antibiotics in controlling *A. hydrophila* infection.

Introduction

Aeromonas hydrophila is a ubiquitous gram-negative bacterium in the aquatic environment that affects both cultured and feral fisheries, inflicting severe economic losses. As a primary pathogen, it causes severe ulcerative dermatitis leading to motile aeromonad septicemia (Hayes, 2000) and, as a secondary pathogen, it is implicated in the devastating epizootic ulcerative syndrome (Thampuran et al., 1995).

Chemicals such as malachite green, formalin, sodium chloride, sulphonomide, nitro-

* Corresponding author. Tel.: +91-431-2407045, +91-431-2407040, e-mail: bal213@rediffmail.com

furan derivatives, and pyridinecarboxylic acid have been commonly applied to control fish diseases with partial success. Habitual use of antibiotics and chemicals as the most widely adopted disease management strategy has resulted in the emergence of antibiotic resistant strains and the accumulation of unacceptable residues in the environment and aquaculture products. For example, an antibiotic resistant strain of *A. hydrophila* was isolated from the skin, organs, and intestinal tract of the mirror carp, *Cyprinus carpio* (Bornemann, 1989). The isolates were resistant to 50 mg/l ampicillin, 30 mg/l kanamycin, and 20 mg/l chlortetracycline. Similar reports on fishes and shrimp are available.

Aeromonas hydrophila is a persistent problem in gold fish farms. A number of carp farms have been closed due to *A. hydrophila* infection in India, where carps form a major part of the diet of poor people. Hence, the outbreak of diseases such as motile aeromonad septicemia or epizootic ulcerative syndrome affects not only the income of marginal fish farmers but also the protein malnutrition that is already prevalent among lower income groups.

Alternative methods, such as phytotherapy, can be additional tools in fish disease management. In India, 500 medicinal plant species are used to treat diseases in humans. Plants have been used as traditional medicine since time immemorial to control bacterial, viral, and fungal diseases. Recently, research has been initiated to evaluate the feasibility of using herbal medicines in fish disease management (Abutbul et al., 2005).

Plant medicines have minimal side effects, are easily biodegradable, inexpensive, and locally available, and extracts are easily prepared. Therefore, it would be beneficial to use herbal extracts as an alternative tool for bacterial disease management in aquaculture. This study aims to quantify the antimicrobial efficacy of five traditional Indian herbs against the fish pathogen, *A. hydrophila*, *in vitro*.

Materials and Methods

Bacterial strain. The reference strain of *Aeromonas hydrophila*, MTCC code no-646, was purchased from the Institute of Microbial Technology (Government of India) in Chandigarh and maintained in our laboratory under standard conditions. Subcultures were maintained on tryptone soy agar (Hi Media, France) slopes at 30°C and periodically checked for pathogenecity on the basis of whether ulcers occurred in infected animals.

Preparation of herbal extract. Plants of *Acalypha indica, Coleus aromaticus, Heliotropium indicum*, and *Indigofera aspalathoides* were collected from the Bharathidasan university campus. Rhizomes of *Acorus calamus* were purchased from local shops that sell traditional medicines. The herbs were washed separately in running water for 3 min and completely shade dried at room temperature (30±2°C) for one week until weight constancy was achieved. Extracts were prepared from powdered leaves of *A. indica*, *C. aromaticus*, and *H. indicum* with 90% w/w ethanol using a soxhlet apparatus (Riviera Glass Pvt. Ltd., Mumbai, India). The ethanol in the extract was completely removed under pressure for 30 min using a rotary evaporator and the dry residues were stored in a dark bottle at 4°C.

Preparation of test solution and inoculum. Six mg of dry extract from each plant were diluted with sterile water to obtain test concentrations ranging 0.05-6 mg/ml. *Aeromonas hydrophila* was incubated in 100 ml Mueller Hinton broth for 18 h at 30°C and then centrifuged at 10,000 rpm for 20 min at 4°C. The supernatant was discarded and the bacterial pellets were washed with phosphate buffer saline (7.2 pH), adjusted to 2.7 x 10^3 cfu (colony forming units) using a Neubauer hemocytometer and incubated for 18 h. The number of cfu was counted using digital counting chambers (ELICO Ltd, India). The autoclaved agar medium was taken in a 200 ml sterilized conical flask into which the serial diluted test solutions were added separately at 18ºC and mixed thoroughly by manual shaking. Twenty ml each of this mixture was poured into ten groups of petri dishes in triplicate (10 x 3 = 30). After solidification of the mixture, 0.05 ml inoculum was added to the petri dish.

For comparison, a positive control of terramycin was maintained separately under similar conditions at the same test concentrations. Terramycin is the most potent antibiotic against *A. hydrophila* used in aquaculture disease management, more potent than chloramphenicol, florfenicol, tetracycline, and streptomycin (Swann, 1989; Hayes, 2000). Inoculated agar plates pre-soaked with ethanol served as the negative control.

Determination of minimum inhibitory concentration (MIC). The minimum inhibitory concentration (MIC) is the smallest concentration of antibacterial compound that prevents development of visible growth of a pathogen. MIC was quantified as the number of cfu on agar plates, following the viable counting method of Cappuccino and Sherman (1997), after incubation for 24 h at 30°C.

Determination of minimum bacterial concentration (MBC). The minimum bacterial concentration (MBC) is the lowest concentration of antibacterial agent that allows less than 0.1% of the original inoculum to survive. MBC was measured by the standard well-in-seeded-plate method (Perez et al., 1990). *Aeromonas hydrophila* cultures were rejuvenated and streaked onto agar plates (n = 3) under sterile conditions. Wells (n = 3) were dug with a cup-borer (diameter 6 mm) into which 40 µl of the test solution was added containing 0.77 mg/ml *A. calamus* or *I. aspalathoides* or 1.29 mg/ml *A. indica*, *C. aromaticus, H. indicum*, or terramycin. The plates were incubated at 30°C for 24 h and the zones of inhibition surrounding the wells were measured to the nearest 1 mm.

Statistical analysis. MIC and MBC means±SD of the treatments and controls were compared using the Students *t* test of the COSTAT statistical package.

Results

The weight of the residue obtained from 100 g plant is indicated in Table 1. The percent extract is the ratio between the dry weight of the plant and the dry weight of the residue. Hence, the percent extract decreased as the weight of the residue increased, indicating variable quantities of ethanol soluble fractions in the chosen herbs.

Minimum inhibitory concentrations (MIC). The MIC for *A. calamus* and *I. aspalathoides* were 1.29 and 2.16 mg/ml, respectively (Table 2). The MIC was not reached for *C. aromaticus*, *A. indica, H. indicum*, and the positive control, even at the highest concentration of 6 mg/ml. The antibacterial activity of *A. indica* and *H. indicum* were almost identical at concentrations of 0.46-0.16 mg/ml. The highest value was recorded in the negative control.

Minimum bacterial concentrations (MBC). The MBC value was determined from the viable counting method (Table 3). The concentrations of 0.77 mg/ml of *A. calamus* and 1.29 mg/ml of *I. aspalathoides* yielded superior effects ($p<0.05$).

Table 1. Quantity of residue (dry wt) and percent extract (wt herb/wt extract) from 100 g (dry wt) of experimental herb.

Herb	*Residue (mg)*	*wt/wt (%)*
Acalypha indica	4.20	22.95
Acorus calamus	4.36	26.45
Coleus aromaticus	3.78	25.25
Heliotropium indicum	3.96	22.57
Indigofera aspalathoides	4.43	23.80

Table 2. Number of colony forming units of *Aeromonas hydrophila* (cfu±SD) on agar plates treated with different concentrations of medicinal plant extracts or terramycin as a positive control. Minimum inhibitory concentration (MIC) is reached when cfu = 0.00±0.0. MIC for negative control (ethanol) was 967.0±3.0 cfu.

Concentration (mg/ml)	*Positive control*	*Acalypha indica*	*Acorus calamus*	*Coleus aromaticus*	*Heliotropium indicum*	*Indigofera aspalathoides*
6.00	2.33±0.7	5.6±0.3	0.00±0.0	4.33±0.33	10.0±1.1*	0.00±0.0
3.60	9.76±1.7	13.9±0.8	0.00±0.0	7.03±0.6	22.5±1.4**	0.00±0.0
2.16	12.00±2.0	22.3±1.4**	0.00±0.0	14.6±0.8	35.0±1.7**	0.00±0.0
1.29	16.33±2.7	53.9±1.7**	0.00±0.0	18.3±9.0	68.5±4.7**	1.00±0.0**
0.77	20.67±3.7	85.5±2.0**	1.00±1.0*	36.2±1.7*	102.0±6.0**	12.66±4.3
0.46	24.00±4.1	224.6±6.9***	14.3±1.2	69.7±7.5**	263.6±3.4***	56.2±5.1*
0.27	62.3±5.17	404.6±1.0***	57.3±6.9	156.8±6.9**	401.6±5.7***	89.8±7.0*
0.16	128.0±0.5	577.3±4.2***	114.0±5.5*	182.1±6.9***	630.3±7.3***	154.6±11.5
0.09	134.6±1.7	606.3±9.0***	128.0±2.3	306.0±1.4***	793.6±3.0***	170.7±8.3*
0.05	155.0±2.0	680.0±0.3***	151.0±1.7	346.3±23.0**	943.6±5.2***	235.6±5.0**

Significant difference from positive control: * $p<0.05$; ** $p<0.01$; *** $p<0.0001$

Table 3. Minimum bacteria concentrations (MBC) and zones of inhibition of herbal extracts and positive control (n = 3). Negative control produced no zone of inhibition.

Herb	*MBC (mean±SD)*	*Zone of inhibition (mm)*
Acalypha indica	53.91±1.75	6.66±1.41*
Acorus calamus	00.00±0.00	13.3±0.91*
Coleus aromaticus	26.16±9.05	8.3±1.44
Heliotropium indicum	68.50±4.74	4.3±0.57**
Indigofera aspalathoides	1.00±0.00	10.0±0.91
Terramycin (positive control)	7.33±2.79	9.17±1.22

* $p<0.05$
** $p<0.01$

Discussion

The *A. calamus* extract inhibited the *in vitro* growth of *A. hydrophila* better than the other four extracts and the positive control at all tested concentrations,. The antimicrobial properties (MIC) of other medicinal plants against the *in vitro* growth of *A. hydrophila* are presented in Table 4. *Acorus calamus* has the lowest value of all the reported herbs. *Acorus calamus* and *I. aspalathoides* are traditionally used in India as medicinal plants for treatment of bacterial infection (Panchal et al.,1989). These plants possess major imminent medicinal properties (Vohora et al., 1990; Rahman and Shereen, 2002) and many commercial drugs have been prepared from them (Lai et al., 2002; Jiang et al., 2005; Ka et al., 2005). In humans, *A. calamus* is used as a curative against skin diseases (Jager et al., 2005). Its antifungal property has been documented (Qureshi, 1997; Begum et al., 2004) and essential *A. calamus* oil has antibacterial and anti-inflammatory activity (Svoboda and Hampson, 1999). *Indigofera aspalathoides* possesses anti-tumor (Rajkapoor et al., 2004) and anti-inflammatory properties (Amala et al., 1982).

Herbal extracts have specific inhibitory potency against specific pathogens. The mode of extraction is also important with reference to antimicrobial properties since solvents are selective in extracting compounds. Avirutnant and Pongpan (1983) found that alcohol extracts of *Cassia alata* were not effective against *Staphylococus aureus, Escherichia coli*, and *Salmonell typhi* but Crockett et al. (1992) found that aqueous extracts had marked activity against infections caused by *Candide albicans* and dermatophytes. Methanol extracts from the leaves of *Azidirachta indica* have moderate activity against *S. typhi B* and *E. coli* whereas the hexane extract was not active against either (Aziz Ullah et al., 2003). In some herbs, the aqueous and ethanol crude extracts exhibit similar antibacterial activity. Harikrishnan (2003) showed that the aqueous and ethanol extracts of chosen herbs have equal effects against *A. hydrophila in vitro*.

Terramycin did not inhibit *A. hydrophila*, even at the highest concentration of 6 mg/ml. Yet two plants totally inhibited the growth of *A. hydrophila* at even lower concentrations. The results indicate the superiority of ethanol extracts of *A. calamus* and *I. aspalathoides* in controlling *A. hydrophila in vitro*.

The quantity of obtained residue varies with the species, the plant part used, and the mode

Table 4. Effective MIC concentration (mg/ml) of ethanol extracts of different herbs used against *A. hydrophila in vitro.*

Herb	*Area*	*Concentration (mg/ml)*	*Reference*
Acorus calamus	India	0.77	Present work
Acalypha indica	India	7.00	Present work
Andrographis paniculata	Thailand	2.50	Direkbusarakom, 1998
Azadirachta indica	India	5.00	Harikrishnan, 2003
Cassia alata	Thailand	*	Harikrishnan, 2003
Clinacanthus nutans	Thailand	*	Harikrishnan, 2003
Coleus aromaticus	India	6.50	Present work
Curcuma longa	India	7.00	Harikrishnan, 2003
Eclipta alba	Thailand	2.50	Direkbusarakom, 1998
Heliotropium indicum	India	10.00	Present work
Indigofera aspalathoides	India	2.16	Present work
Mixture	India	5.00	Harikrishnan, 2003
Momordica charantia	Thailand	2.50	Direkbusarakom, 1998
Ocimum sanctum (red)	Thailand	*	Direkbusarakom, 1998
O. sanctum (white)	Thailand	*	Direkbusarakom, 1998
O. sanctum	India	10.00	Harikrishnan, 2003
Phyllanthus acidus	Thailand	*	Direkbusarakom, 1998
P. amaras	Thailand	*	Direkbusarakom, 1998
P. debilis	Thailand	10.00	Direkbusarakom, 1998
P. pulcher	Thailand	*	Direkbusarakom, 1998
P. reticulates	Thailand	2.50	Direkbusarakom, 1998
P. arinaria	Thailand	2.50	Direkbusarakom, 1998
Psidiair guajava	Thailand	0.63	Direkbusarakom, 1998
Tinospora cordifolia	Thailand	10.00	Direkbusarakom, 1998
T. caspa	Thailand	10.00	Direkbusarakom, 1998

* Not effective even at the highest concentration (10 mg/ml) tested.

of extraction. The percent extract (w/w) obtained from the five herbs is ranked *I. aspalathoides* > *A. calamus* > *A. indica* > *H. indicum* > *C. aromaticus*. However, the percent extract has no implication on the antimicrobial properties of the extracts since the MBC of the herbs is *A. calamus* > *I. aspalathoides* > *Coleus aromaticus* > *Acalypha indica* > *Heliotropium indicum*. Work to elucidate the active phytochemical compounds in *A. calamus* and *I. aspalathoides* is underway. Further studies are also being carried out to test the efficacy of the herbal extracts in fish models *in vivo*.

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